Amendments to the Specification:

(Page 1, lines 5-6) The work leading to this invention was supported in part by a grant from NIH-MCI, Grant No. 1U01CA91178-03. The United States has ~~may have~~ certain rights in this invention.

(Page 3, lines 83-84) Fig.3 shows a saturation curve for GM-BODIPY as different concentrations of Hsp90 alpha. The signal was recorded at the presented time intervals.

(Page 4, line 112) Fig. 23 shows the antimitotic activity of Hsp90 ~~inhibitos~~ inhibitors in MDA-MB-468 cells.

(Page 6, Lines 163 -166) As used in the specification and claims of this application, the term binding range refers to the difference (maximum mP at saturation-minimal mP at no protein). In an optimized fluorescence polarization assay, the binding affinity of the probe to the protein should be high and the binding range should be large.

(Pages 7-8, Lines 202-230) Suitable fluorescently-labeled molecules include those molecules known to bind to Hsp90 which can be modified to incorporate a fluorescent label without interfering with the binding of the molecule to Hsp90. For example, in the case of geldanamycin, modification at C17 to incorporate a fluorescent label such as FITC or BODIPY results in a labeled binding agent which can be used in the assay of the invention. A synthetic scheme for the preparation of such ligands is shown in FIG. 1 and described in Example 1 below. Other suitable fluorescently-labeled molecules can be based on radicicol, and small molecules inhibitors that are discussed above. The amount of Hsp90 employed is sufficient to produce a detectable fluorescence signal. As can be seen in the examples, maximum levels of polarization were

observed at Hsp90 alpha concentrations of 50 to 75 nM at constant concentration of the fluorescently-labeled geldanamcyin. While the absolute concentrations may vary from one fluorescently-labeled molecule to another, and from ~~on~~ one Hsp90 to another, the assay is desirably performed under conditions, where the degree of polarization observed in the absence of the candidate molecule is substantially independent of the amount of hsp90 present. When using cell lysates instead of recombinant protein, the assay measures binding to average Hsp90 population found in cell specific complexes, be it transformed or normal cell. Suitable cells include all transformed cells in which transformation is driven by an event regulated by hsp90. Specific cell types that are suitably used include, without limitation, mammalian (include human and murine) breast cancer cells (ex. SKBr3, MCF7, MDA-MB-468, BT-474), glioblastoma cells(U87), neuroblastoma cells (ex. SH-SY5Y, N2a), vulvar cancer cells (ex. A431), small cell lung cancer cells (ex. NCI-N417, NCI-H69), prostate cancer cells (ex. LAPC, LNCaP, PC3, TSuPrl), acute myeloid leukemia cells (ex. Kasumi-1, NB4), acute promyelocytic leukemia cells, chronic myeloid leukemia cells (ex. K562), colon cancer cells (ex. Colo205), non-small cell lung cancer cells (ex. A549), melanoma cells (ex. SKMel28), pancreatic cancer cells (ex. AsPC-1, BxPC-3, Capan-2, Miapaca-2, and Panc-1) and normal brain, liver, kidney, pancreas, spleen, lung and heart cells Normal tissue/organs that are suitably used include, without limitation, normal brain, liver, kidney, pancreas, lung and heart cells. Compounds with highest affinity and selectivity for transformed cell hsp90 are favored.

(Page 8, Lines 231-233) The second assay method of the invention ~~of the invention~~ is a cell based assay for identifying a candidate molecule as having activity as an inhibitor of Hsp90. The method comprises the steps of:

(Page 9, Lines 248-260) In one embodiment of the invention the amount of an Hsp90-dependent protein is determined using an immunoassay. One such immunoassay methodology is similar in

concept to a western blot assay or a cytoblot assay of the type described by Stockwell et al. *Chemistry* and *Biology* 6: 71-83 (1999). These assays, however, test for the presence of a given species. In the second assay, that which is being tested for is the absence or near absence of a chemical species. Testing for the presence of a gene product is far simpler than testing for the absence of a gene product since the amount of gene product presumably increases with time in the first instance, and thus distinguishes itself from any background measurements. In contrast, when the disappearance of a product is measured, the best resolution from background is at time zero, when the assay has not yet started. Surprisingly, notwithstanding this theoretical difficulty, meaningful observations can be made of the loss of Her2 and EGFR expression in the presence of an Hsp90 inhibitor, thus providing an immunoassay which can be used to screen ~~candidtate~~ <u>candidate</u> molecules for activity as inhibitors of Hsp90 and inducers of Her2 and EGFR degradation.

(Pages 13-14, Lines 384-405) To rule out that the declining signal in drug treated cells was not the result of reduced cell number caused by unspecific cell death but to decreased Her2 content, we determined the amount of total protein by the bicinchoninic assay (BCA) (Smith, et al. "Measurement of protein using bicinchoninic acid". Anal. Biochem. 50, 76-85, ~~19985~~ <u>1985</u>) and additionally, tested changes in 0-actin protein levels. We found the BCA assay to be compatible with the Her2-blot reagents. Two 96-well plates were treated with various concentrations of PU24FCl; the first was subjected to a BCA assay alone, and the second to a Her2-blot experiment followed by a BCA assay. Measured differences in total protein between the two plates were insignificant and an average of 1.5-1.6 g protein was detected in each well. We additionally monitored possible changes in 0-actin levels due to drug addition, prior to and following the Her2-blot. If the 0-actin-blot was performed subsequent to the Her2-blot, plates were stripped and blocked for nonspecific binding prior to the addition of an anti-actin antibody. As expected, the levels of actin in the stripped plate were slightly diminished but detectable.

Thus this method, similar to western blot, allows for re-blotting of the plate. No significant differences in actin expression were seen between untreatedand Her2-depleted cells validating the observation that the lower Her2 levels in treated cells were indeed due to a selective reduction in oncoprotein levels. If, however, variation in total protein content is observed, values obtained for Her2 can be normalized to total protein concentration. Both the BCA assay and -actin-blot are compatible with the Her2-blot and can be performed following Her2 quantification. It is noteworthy that actin-level measurements could be indicative of the antiproliferative effect of the tested compounds. Thus in addition to identifying changes in Her2, screening would be indicative of cytotoxic effects of the compounds.

(Pages 14 & 15, Lines 419-426) While in its simplest form the second type of assay can be performed using a single transformation-specific cellular "fingerprint" of Hsp90 inhibition, its ~~is~~ desirable to use multiple transformation types to provide a more detailed cellular "fingerprint" of Hsp90 inhibition which can show a wider scope of therapeutic applicability for a given candidate molecule. To provide such a detailed fingerprint, test cells are suitably selected to provide information about diverse Hsp90-dependent activities. Thus, in one embodiment of the invention, a candidate molecule is screened using any combination of two or more of the following cell types, and preferably all four:

(Page 15, Lines 429-430) (2) cells with Raf-MAPK driven transformation, as exemplified by MCF7 breast cancer cells that are Rb', ER' cells with high levels of Rafl; determine level of Raf-1.

(Page 15, Lines 431-432) (3) cells which are PTEN-defective cells with high Akt levels/activity, as exemplified by U87 glioblastoma; determine level of Akt.

(Page 17, Lines 498-514) GM-FITC: To a solution of FITC-NCS (78 mg, 0.20 mmol) and 6-(Boc-amino)-1-hexanol (43 mg, 0.20 mmol) in DMF (0.75 mL) at 60° C. was added TEA (16 L, 0.4 mmol). After it was heated with stirring for 24 h, the solution was cooled to room temperature, and the solvent was removed in vacuo. Silica gel column chromatography with DCM/hexanes/MeOH (6:4: 1) afforded the corresponding thiocarbamate (60 mg, 50%) as an orange crystalline solid. 1H NMR (400 MHz, MeOH-d4): 7.33-7.25 (m, 6H), 7.21-7.16 (m, 4H), 5.76-5.68 (m, lH), 5.05 (d, J=17.1 Hz, lH), 5.01 (d, J=10.2 Hz, lH), 4.51-4.48 (m, lH), 4.05 (dd, J=1.6, 9.0 Hz, lH), 3.98 (t, J=8.4 Hz, lH), 3.44-3.36 (m, 2H), 3.25 (dd, J=3.1, 14.0 Hz, lH), 3.20 (dd, J=3.3, 13.4 Hz, lH), 2.66 (dd, J=9.8, 13.4 Hz, lH), 2.48-2.44 (m, 2H). MS m/z 608.1 (M+H). To the thiocarbamate (7 mg, 0.012 mmol) was added <u>to</u> a mixture of CH2C12/TFA (0.3 mL/O.1 mL) and the resulting solution was stirred at room temperature for 45 min. The solvent was removed in vacuo. The crude was taken up in DMF (0.5 mL) and GM (6.4 mg, 0.012 mmol) and an excess triethylamine (50 L, 36 mmol) were added to the solution. The mixture was stirred under inert gas atmosphere for 24 h. After solvent removal in vacuo, the product was purified on a silica gel column eluting with CH2C12/EtOAc/hexanes/MeOH (4:2:3:1) to afford GM-FITC (5.5 mg, 44%) as a yellow-orange solid.

(Page 18, Lines 520-531) To assess the suitability of these probes for Hsp90 in a homogenous FP assay format using an Analyst AD (Molecular Devices) instrument, a stock of 10 M of each tracer was prepared in DMSO and diluted with HFB buffer (20 mM HEPES (K) pH 7.3,50 mM KCl, 1 mM DTT, 5 mM MgCl2, 20 mM Na2Mo04, 0.01% NP40 with 0.1 mglmL BGG) to obtain 10 nM and 4 nM solutions for GM-BODIPY and GM-FITC, respectively. Different amounts of Hsp90 alpha (Stressgen # SPP776) dissolved in HBF were added to a low binding black 96 well-plate (Coming # 3650) in a 50 L volume. To each well were added 50 L of the tracer solution. Some wells were left with buffer or tracer alone to serve as controls. The plate was left on a shaker at 4° C. for 3 h and the FP values in mP were recorded. The measured FP

value (mP) was plotted against the protein concentration (FIG. 2). Both tracers performed well in the assay. The titration curve showed that the probes bind tightly to Hsp90. The dynamic binding range of FP was approximately 160 mP.

(Page 18-19, Lines 538-555) Competitive displacement studies were performed with known Hsp90 inhibitors 17AAG and PU3 (structure shown in FIG. 6) and additionally as a control, with Ad-But, a PU3 derivative that does not bind Hsp90 (Schulte, et al. "The benzoquinone ansamycin 17-allylamino-17- demethoxygeldanamycin binds to Hsp90 and shares important biologic activities with geldanamycin." Cancer Chemother. Pharmacol., 42: 273-279, 1998; Chiosis, et al., "A small molecule designed to bind to the adenine nucleotide pocket of Hsp90 causes Her2 degradation and the growth arrest and differentiation of breast cancer cells."Chem. Biol., 8: 289-299, 2001). Stocks of these molecules were made in DMSO at concentrations of 200 M for 17AAG and 4 mM for PU3 and Ad-But. The drugs were serially diluted in binding buffer and the GM-BODIPY tracer and Hsp90 were added at 5 nM and 40 nM concentrations, respectively. Maximum concentration of used DMSO was 0.25% (v/v). The plate was left on a shaker at 4° C. for 5 h and the FP values in mP were recorded. A window of 100 mP was observed between wells containing protein and tracer and wells containing tracer only. The measured FP values (mP) were plotted against the competitor concentration (FIG. 4). EC50 values were determined as the competitor concentrations ~~were~~ where 50% of the tracer was displaced. As previously reported, PU3 was found to be a 30-times weaker Hsp90 inhibitor than 17AAG (EC50=3.2 M vs 110 nM). Ad-But could not displace Hsp90 bound GM-BODIPY even at the maximally measured concentration of 20 μM.

(Page 20, Lines 587-594) Competitive binding experiments of GM with added 0, 2, 4, 8 and 16% DMSO (v/v) were performed in the presence of 5 nM fluorescent GM and 30 nM Hsp90 . Data from the binding results were analyzed using Prism 4.0. (FIG. 9A) Data recorded in tracer only wells were subtracted from control wells (no GM present) and plotted as a function of DMSO

concentration to present the effect of the organic solvent on the assay window. The assay window was defined as mP-mPf. (FIG. 9B). Values recorded in wells with added GM were normalized to data in control wells and plotted against the concentration of tested GM for each DMSO concentration.

(Page 20-21, Lines 596-602) Twenty 96-well plates each containing four free tracer control wells (5 nM GM-BODIPY) and four bound tracer control wells (5 nM GM-BODIPY with added 30 nM Hsp90) were used to determine the suitability of the assay for HTS (all other wells contained compounds tested for binding to Hsp90). Each plate corresponds to an assay conducted on a different day. The mP value for each well was recorded and average values corresponding to each plate were plotted. (FIG. 10A). The signal-to-noise ratios and the Z' factors were calculated for each plate. (FIG. 10B).

(Page 26, Lines779-786) FIGS. 16A and B show results of assay optimization and performance testing using the SKBr3 cells. For ~~evaluate~~ evaluating the optimal cell number, SKBr3 cells were left to attach for the indicated time period prior to Her2-blot analysis. The signal recorded in anti-Her2 (S) and IgG (B) reacted wells was recorded and the ration SIB was plotted as a function of cell number (FIG. 6A). Each measurement represents the average of 8 wells. Results of Z'-analysis in the Her2 blot are shown in FIG. 16B. SKBr3 cells were reacted with the anti-Her2 antibody (open circles; std= 7.5%), the corresponding IgG (solid squares; std=16.8%) and twenty Hsp90 inhibitors [at 30 M] from an in-house library (solid triangles).

(Page 28, Lines 819-829) To confirm the utility of the assay in a library screen format to identify agents capable of altering the cellular levels of these kinases, a subset of our Hsp90-inhibitor library was added to SKBr3 cells at a set concentration of 30 μM and the ability of these agents

to decrease Her2 levels in cells was determined after a 6 h treatment. Agents that alter cellular levels of kinase by at least 25-30% can be considered "hits" with a high degree of confidence. There was no significant change in total protein content as determined by the BCA assay and thus, the values reflect the effect of these compounds on Her2 expression. The ~~result~~ results are summarized in FIG. 20. The average signal obtained for each of these agents (in RLU) is plotted as the solid triangles in FIG. 16B. It is noteworthy to mention that identical activity in the series of compounds was previously obtained using the traditional western blot, however this work required a 3-week period.

(Page 30, Lines 868-880) Following the 24-hour incubation, the wells were washed twice with ice-cold lx TBST (Tris Buffer Saline containing 0.1% Tween 20). A house vacuum source attached to an eight-~~channle~~ channel aspirator was used to remove the liquid from the 96-well plates. Then, 100 µL of ice-cold methanol was added to each well, then placed in 4 C for 5 min. After 5 min, methanol was suctioned out, and the plates were washed three times with ice-cold lx TBST (2x200 µL). After the removal of methanol, the plates were further incubated with SuperBlock® blocking buffer (Pierce #37535) for 2 hours at RT. Then TG3 antibody was diluted 1:200 in SuperBlock®, and placed in each well (100 µL) except the first column; the first column was treated with Ig control (Neg. Control for Mouse IgM, NeoMarkers, NC-1030-P), 1:200 dilution in Superblock®. After 72 hours, all wells were washed with ice-cold lx TBST twice, then secondary antibody (Goat Anti-Mouse IgM, SouthernBiotech, 1020-05) in 1:2000 dilution in SuperBlock® was placed in each well, and incubated on a shaker at RT for 2 hours.

(Page 30, Lines 889-892) FIG. 23 shows the antimitotic activity of Hsp90 ~~inhibitos~~ inhibitors in MDA-MB-468 cells. As shown MDA-MB-468 cells were treated for 24 h with several small

molecules. The percentile increase in cells in mitosis compared to untreated cells was quantified using a p-nucleolin specific antibody.